

Bioengineering of Human Thyrotropin Superactive Analogs by Site-directed “Lysine-scanning” Mutagenesis

COOPERATIVE EFFECTS BETWEEN PERIPHERAL LOOPS*

Received for publication, May 2, 2000, and in revised form, June 14, 2000
Published, JBC Papers in Press, June 19, 2000, DOI 10.1074/jbc.M003707200

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We have previously engineered the first superactive analogs of human thyrotropin (hTSH) by using a novel design strategy. In this study, we have applied homology comparisons focusing on the α L3 loop of the common α -subunit of human glycoprotein hormones. Seven highly variable amino acid residues were identified, and charge-scanning mutagenesis revealed three previously unrecognized modification permissive domains and four gain-of-function lysine substitutions. Such gain-of-function mutations were hormone- and receptor-specific and dependent on location and basic charge. Cooperativity of individual substitutions was established in double and triple lysine mutants. In combinations of the most potent α L3 loop analog with two previously characterized loop analogs, a higher degree of cooperativity for the α L3 loop analog compared with both the α L1 loop analog and the hTSH- β L3 loop analog was observed. We demonstrated that spatially distinct regions of the common α -subunit contribute differentially to the interaction of hTSH with its receptor and that combinations of two modified loops on the same and on opposite sides of the hTSH molecule display similar increases in *in vitro* biopotency. In addition, combination of all three superactive loops showed cooperativity in receptor binding and activation resulting in the most potent hTSH superactive analog described to date.

consists of a heterodimer of an α -subunit and a noncovalently linked β -subunit, and the α - β heterodimer formation is required for full biological activity. Whereas the primary structure of the α -subunit is common to all four glycoprotein hormones, the β -subunit is unique and responsible for hormone specificity (1). More recent studies have suggested a potential role for the individual free subunits in pregnancy and tumor growth (2–4).

The recent elucidation of the crystal structure of chemically deglycosylated hCG (5, 6) has revealed that glycoprotein hormones share structural features with a large number of growth factors such as platelet-derived growth factor, neurotrophin (nerve growth factor), and transforming growth factor β (7) and are therefore considered members of the superfamily of cystine knot growth factors. Within each glycoprotein subunit, a central cystine knot motif consisting of three disulfide bonds can be identified together with two peripheral β -hairpin loops (L1 and L3) on one side and one long loop (L2) on the other side. Despite a large diversity of membrane receptors within the superfamily of cystine knot growth factors, there are marked similarities in the functional role of the peripheral loop segments despite a limited (approximately 10–15%) amino acid sequence homology between the glycoprotein hormones and other members of the superfamily. Specifically, clusters of positively charged residues within the loops have recently been implicated as important receptor binding domains for members of the neurotrophin and the transforming growth factor β family (8, 9).

Recent structure-function studies of human thyrotropin from our laboratory (for reviews see Refs. 10–12) and others have characterized multiple functionally important domains in both subunits (Fig. 1). We distinguished “specificity determining” domains in the hTSH β “seat belt” (hTSH β (88–105) (13)), “modification non-permissive” (α 33–38 (14), α -helix α 40–46 (14, 15), glycosylation site α 52 (16, 17), α -carboxyl terminus α 88–92 (18–20)), and “modification permissive” domains in two peripheral β -hairpin loops (α 11–20 (21), hTSH β (58–69) (22)). The mutational analysis of modification permissive domains defined as regions that tolerate the introduction of non-conservative amino acid changes into hTSH without compromising hormone synthesis (10) has been particularly useful in recent structure-function studies.

Based on evolutionary considerations, amino acid structure comparisons between species and between different glycoprotein hormones, as well as computer-assisted homology modeling, we were able to define a design strategy for bioengineering superactive analogs (“superagonists”) of human thyrotropin with major increases in both receptor binding affinity as well as

Human thyrotropin (hTSH)¹ is a member of a family of structurally related pituitary and placental human glycoprotein hormones that also includes the gonadotropins human chorionic gonadotropin (hCG), human-luteinizing hormone (hLH), and human follicle-stimulating hormone. Each hormone

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¹ The abbreviations used are: hTSH, human thyrotropin; bTSH, bovine thyrotropin; hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; TT4, total thyroxine; PCR, polymerase chain reaction; CHO, Chinese hamster ovary.

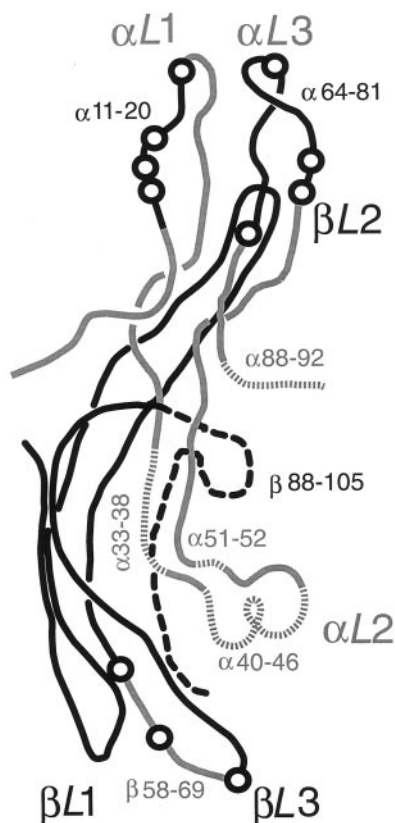


FIG. 1. **Schematic drawing of hTSH.** Schematic drawing of hTSH depicts the α -subunit in gray and the β -subunit in black. Functionally critical domains are marked directly within the line drawing (for more details, see text). The peripheral β -hairpin loops are indicated as α L1 and α L3 in the α -subunit and as β L1 and β L3 in the β -subunit. Modification permissive domains are the regions between α 11 and 20 in the α L1 loop and β 58 and 69 in the β L3 loop. The region between α 64 and 81 was the target region for site-directed mutagenesis in this study. Open circles represent the positions of individual amino acid residues that were substituted with basic residues (α 13, α 14, α 16, and α 20 in α L1; α 64, α 66, α 73, and α 81 in α L3; β 58, β 63, and β 69 in hTSH β L3). Modified from Ref. 11.

signal transduction which were the first to be described for any glycoprotein hormone or any member of the cystine knot growth factor superfamily and which by far exceeded the increases in biopotency obtained for other protein ligands such as growth hormone (23) or interleukin-6 (24) using empirical design approaches. In contrast to the characterization of loss-of-function mutations, which could result in distant conformational effects leading to an indirect alteration of hormone function, the analysis of gain-of-function mutations is expected to be much more specific with respect to their location and the underlying mechanism and therefore more informative in glycoprotein structure-function studies.

In the present study, we have extended our design strategy for bioengineering superactive analogs of human thyrotropin to the second, as yet uncharacterized peripheral β -hairpin loop of the common α -subunit of human glycoprotein hormones (*i.e.* the α L3 loop, Fig. 1) under the assumption that introduction of positively charged, basic residues into all four peripheral β -hairpin loops leads to enhanced electrostatic interactions with the hTSH receptor resulting in an increase in hormone binding affinity and in *in vitro* biopotency. The aims of the present study were as follows: 1) to identify modification permissive domains within the common α L3 loop of human glycoprotein hormones; 2) to identify amino acid residues where substitutions to basic residues lead to the generation of superactive hTSH analogs; 3) to combine single α L3 loop mutations

to study cooperative effects between individual residues within this loop; and 4) to study cooperative effects between the α L3 loop and other peripheral β -hairpin loops.

EXPERIMENTAL PROCEDURES

Materials—Molecular biology reagents were purchased from Life Technologies, Inc., Roche Molecular Biochemicals, and New England Biolabs, Inc. (Beverly, MA). Cell culture media, media supplements, and the LipofectAMINE reagent were supplied by Life Technologies, Inc. The full-length human α -cDNA subcloned into the *Bam*HI-*Xho*I restriction enzyme sites of the pcDNAI/Neo expression vector (Invitrogen, San Diego) was obtained from T. H. Ji (University of Wyoming, Laramie). The hTSH β -minigene in the pLBCMV vector was previously engineered in our laboratory (25). Chinese hamster ovary (CHO) cell clones stably expressing the hTSH wild-type receptor (JP09 and JP26) at different receptor densities were kindly provided by G. Vassart (Free University of Brussels, Brussels, Belgium). In addition, another CHO cell clone (hTSHR-D1) has been established using a construct supplied by B. Rapoport (Veterans Affairs Medical Center, San Francisco, CA) (26), in which amino acid residues 317–366, unique for the hTSH receptor, have been deleted. This construct was used to study the role of TSH receptor-specific structures in the observed increase in hTSH analog bioactivity. cAMP antibody was a gift from J. L. Vaitukaitis (National Institutes of Health, Bethesda). 125 I-cAMP and 125 I-hTSH were purchased from Covance Laboratories Inc. (Vienna, VA).

Site-directed Mutagenesis—Mutagenesis of the human α -cDNA was accomplished by the PCR-based megaprimer method of site-directed mutagenesis with two consecutive PCR cycles as described previously (27), using Vent DNA Polymerase (New England Biolabs, Inc., Beverly, MA) and oligonucleotide primers synthesized by Lofstrand Laboratories Ltd. (Gaithersburg, MD) with the wild-type human α -cDNA in the pcDNAI/Neo vector as template. For generation of multiple mutations within the α L3 loop, various previously mutated α -cDNAs in the pcDNAI/Neo or pcDNA 3 expression vector (Invitrogen) with single or double amino acid substitutions were used as templates for PCR. Flanking oligonucleotide primers and amplification conditions had been optimized in the course of our previous studies (21). Mutagenizing primers were designed to introduce the following codon changes: S64K (TCA-AAA), Y65K (TAT-AAA), N66K (AAC-AAG), R67K (AGG-AAG), G73K (GGT-AAA), F74K (TTC-AAA), and A81K (GCG-AAG). Additional primers were used to introduce negatively charged residues as follows: S64E (TCA-GAG), N66E (AAC-GAG), G73E (GGT-GAG), G73D (GGT-GAC), and A81E (GCG-GAG). After simultaneous digestion of the product of the second PCR with *Bam*HI and *Xho*I and isolation of the resulting fragment, ligation of this fragment into the wild-type human α -cDNA in the pcDNA3 vector with the *Bam*HI-*Xho*I fragment excised was performed. Combination of the α L1 loop analog with the α L3 loop analog was achieved by single restriction enzyme digest of the α L3 loop analog with *Xba*I and consecutive ligation of the isolated fragment into the α L1 loop analog in the pcDNAI/Neo expression vector with the *Xba*I fragment excised. Chemically competent *Escherichia coli* cells (DH5 α Subcloning Efficient Cells, Life Technologies, Inc., or MC1061/p3, Ultracom Transformation Kit, Invitrogen) were transformed using a heat-shock protocol. Plasmids were isolated using the Qiaprep 8 Miniprep Kit and Qiaprep 8 Turbo Miniprep Kit for multiple preparations of small DNA quantities and the Qiagen Plasmid Maxi Kit (Qiagen Inc., Santa Clarita, CA) for purifications of large DNA quantities. Correct introduction of single or multiple mutations was verified by bidirectional single-stranded DNA sequencing performed by the Biopolymer Laboratory (University of Maryland School of Medicine, Baltimore).

Transient Expression of Recombinant Hormones—CHO-K1 cells (ATCC, Manassas, VA) were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum, glutamine (4 mM), penicillin (50 units/ml), and streptomycin (50 μ g/ml) at 37 $^{\circ}$ C and 5% CO $_2$. Cells were transiently cotransfected in 60-mm dishes after reaching about 80% confluency with wild-type or mutant α -cDNA in the pcDNA3 vector for single and multiple mutants within the α L3 loop and in the pcDNAI/Neo vector for the α L1- α L3 analog combination, respectively, and wild-type or mutant hTSH β -minigene in the pLBCMV vector using a liposome-mediated approach (LipofectAMINE reagent, Life Technologies, Inc.). After 16 h, the transfected cells were transferred to serum-free medium (CHO-SFM II, Life Technologies, Inc.). In a typical experiment, we used about 10–15 60-mm dishes per mutant and added 6 ml of serum-free medium per dish. After an additional 72 h, cell culture media were harvested, resulting in a total volume of 60–90 ml unconcentrated medium per mutant, which was then first clarified by centrifugation. Second, concentration of this volume by about 50-fold was

achieved using Centriprep 10 concentrators (Amicon, Inc., Beverly, MA), resulting in a final volume of about 1.2–1.8 ml, which was then divided into 0.2-ml aliquots, which were stored at -70°C . These concentrated, unpurified media preparations were then used for all further studies as outlined below. Aliquots were thawed only once before each respective assay.

Immunoassays—Wild-type recombinant hTSH and mutant hTSH analogs were quantitated with four different immunoassays utilizing different monoclonal and polyclonal antibodies. Three third generation “sandwich” assay systems (Nichols Institute Diagnostics, San Juan Capistrano, CA; ICN Pharmaceuticals, Inc., Costa Mesa, CA; DiaSorin Inc., Stillwater, MN) recognizing hTSH immunochemiluminometrically or immunoradiometrically by forming a bridge between a solid-phase coupled monoclonal antibody and a second polyclonal acridinium ester- or ^{125}I -labeled antibody were used. In addition, we performed a polyclonal hTSH radioimmunoassay using antibody (NIDDK anti-hTSH-3) directed against the hTSH β -subunit kindly supplied by the National Hormone and Pituitary Program (Torrance, CA) and using recombinant hTSH provided by the Genzyme Corp. (Framingham, MA) as standard (28).

Receptor Binding Assays—The binding activity of wild-type or mutant hTSH was studied by their ability to displace ^{125}I -radiolabeled bovine TSH (^{125}I -bTSH) from a solubilized porcine thyroid membrane preparation (Kronus, San Clemente, CA) by serial dilutions of wild-type or mutant hTSH using a buffer containing 0.15% NaCl according to the protocol supplied by the manufacturer.

Biological Activity Assays—CHO cells stably expressing the human wild-type thyrotropin receptor (JP09 and JP26) or the thyrotropin receptor construct with deletion of amino acid residues 317–366 (hTSHR-D1) were grown in 96-well tissue culture plates in Ham’s F-12 medium supplemented with 5% fetal bovine serum, glutamine (4 mM), penicillin (50 units/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$) at 37°C and 5% CO_2 . After reaching confluency, cells were incubated for 2 h with serial dilutions of wild-type or mutant hTSH in a modified Krebs-Ringer buffer under low salt conditions where sucrose was added to maintain isotonicity supplemented with 1 mM 3-isobutyl-1-methylxanthine and 0.1% bovine serum albumin (Sigma). The amount of cAMP released into the buffer was measured by radioimmunoassay as described previously (29).

In vivo bioactivity was assessed by a previously validated bioassay (30). Male albino Swiss CrI:CF-1 mice were pretreated over 4 days with drinking water supplemented with 0.3 μg triiodothyronine/ml *ad libitum*. This has been shown (30) to stably and reproducibly suppress endogenous thyrotropin secretion, thereby providing indispensable preconditions for the following study by practically eliminating preexisting interanimal variability. Stimulation of total thyroxine (TT4) levels over this suppressed base-line level was chosen as end point. Six hours after an intraperitoneal injection of hTSH wild-type and selected hTSH analogs, blood was collected by retroorbital sinus puncture, and the animals were sacrificed. Total thyroxine (TT4) serum levels were measured by radioimmunoassay (DiaSorin Inc., Stillwater, MN).

RESULTS

Design of Single and Combined Mutants—Amino acid sequence alignment of the primary amino acid structure of the common αL3 loop of human glycoprotein hormones between amino acid residues $\alpha 64$ and $\alpha 81$ was used to select potential target sites for site-directed mutagenesis (Table IA). The $\alpha 64$ –81 region appeared to be predominantly surface-exposed and distant from the β -subunit based on an hTSH homology model (21) constructed using crystallographic coordinates of hCG (5, 6).

In contrast to the $\alpha 11$ –20 region in the common αL1 loop of human glycoprotein hormones (21), the $\alpha 64$ –81 region in the common αL3 loop does not contain clusters of conserved positively charged amino acid residues. We therefore distinguished residues with lower ($\alpha 68$ –72, $\alpha 75$ –80) versus higher ($\alpha 64$ –67, $\alpha 73$ –74, $\alpha 81$) amino acid structure variability during evolution. For their expected higher tolerance toward introduction of non-conservative amino acid changes, we selected the latter as target sites for site-directed mutagenesis. According to our hypothesis that introduction of nonconservative basic residues at these sites may generate “gain-of-function” analogs, we chose to substitute the wild-type amino acid residues at each of the above positions with single lysine residues. This “lysine-

TABLE I
Amino acid sequence alignment of the common αL3 loop (Table IA) of human glycoprotein hormones between $\alpha 64$ –81; design of single (Table IB) and multiple (Table IC) lysine substitutions

	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81
Human	S	Y	N	R	V	T	V	M	G	G	F	K	V	E	N	H	T	A
Rhesus	-	L	T	-	-	M	-	-	-	S	V	R	-	-	-	-	-	E
Marmoset	-	L	T	-	-	M	-	-	-	S	V	R	-	-	-	-	-	E
Bovine	A	F	T	K	A	-	-	-	-	S	V	R	-	-	-	-	-	E
Equine	A	F	I	-	-	-	-	-	-	N	N	I	-	-	-	-	-	E
Mouse	A	F	T	K	A	-	-	-	-	N	N	A	R	-	-	-	-	E
Rat	-	F	T	K	A	-	-	-	-	N	N	A	R	-	-	-	-	E
Salmon	E	G	E	-	-	V	-	-	D	N	I	-	-	L	T	-	-	E
Bass	-	E	E	T	-	-	A	-	-	N	I	-	-	R	-	-	-	E
Carp	E	V	K	-	-	L	-	-	N	I	D	-	-	L	V	-	-	E

S64K	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y65K	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N66K	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R67K	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G73K	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-
F74K	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-
A81K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K

S64K+N66K	K	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S64K+G73K	K	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-
N66K+G73K	-	-	K	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-
G73K+A81K	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	K
S64K+N66K+G73K	K	-	K	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-
S64K+N66K+A81K	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K
S64K+G73K+A81K	K	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	K
N66K+G73K+A81K	-	-	K	-	-	-	-	-	-	-	K	-	-	-	-	-	-	K
S64K+N66K+G73K+A81K	K	-	K	-	-	-	-	-	K	-	-	-	-	-	-	-	-	K

scanning” approach resulted in seven single lysine substitutions as follows: S64K, Y65K, N66K, R67K, G73K, F74K, and A81K (Table IB).

In a second step, four out of seven mutations were then combined to generate four double, four triple, and one quadruple mutant (Table IC).

Quantitation and Relative Expression Levels—Wild-type thyrotropin and mutant human thyrotropin analogs were quantitated with four monoclonal and polyclonal immunoassays. Based on the most sensitive immunochemiluminometric assay, the yield when following the transient transfection procedure outlined above was about 2000 ng of hTSH/ml of concentrated, unpurified medium for the generation of hTSH wild-type. Relative expression levels as compared with wild-type ranged between 28 and 128% for mutants with single substitutions and between 23 and 49% for mutants with multiple substitutions within the αL3 loop. A comparison of expression levels for single, double, and triple lysine substitutions is given in Table II, and expression levels of mutant hTSH analogs with multiple substitutions in different loops are shown in Table III. Immunological recognition of the mutants was highly comparable between all four assays used, thereby ensuring accurate quantitation and providing preconditions for reliable calculation of relative increases in biopotency in the following studies. In the course of these (data not shown) as well as our previous studies, we have determined several hTSH amino acid residues critical for binding of the respective monoclonal antibodies used in these assays, and we found them to be distinct and not identical between the assays chosen for this study.

Analogs with Single Mutations—Introduction of single lysine substitutions at positions $\alpha 64$ –67, $\alpha 73$ –74, and $\alpha 81$ within the common αL3 loop generated seven hTSH analogs all of which were immunologically recognized and biologically active. Therefore, the introduction of nonconservative amino acid residues did not result in a significant alteration of the overall conformation of the hormone or in a significant impairment of its biological activity, thereby defining these regions as modification permissive domains.

TABLE II
Comparison of relative expression levels of wild-type (WT) hTSH and mutant hTSH analogs for single, double, and triple lysine substitutions in four different immunoassays

The expression level of wild-type hTSH equals 100 for each respective assay.

	ILMA monoclonal		IRMA monoclonal	
	Nichols Institute Diagnostics		DiaSorin Corp.	
WT	100		100	
S64K	72 ± 13		62 ± 18	
N66K	128 ± 25		133 ± 24	
G73K	51 ± 13		39 ± 13	
A81K	28 ± 2		24 ± 2	
S64K/N66K	41 ± 2		38 ± 1	
S64K/G73K	35 ± 5		36 ± 1	
N66K/G73K	39 ± 12		38 ± 2	
G73K/A81K	23 ± 3		22 ± 7	
S64K/N66K/G73K	32 ± 8		34 ± 3	
S64K/N66K/A81K	35 ± 1		28 ± 1	
S64K/G73K/A81K	49 ± 22		42 ± 11	
N66K/G73K/A81K	33 ± 9		25 ± 7	

TABLE III

Comparison of relative expression levels of wild-type (WT) hTSH and mutant hTSH analogs for combinations of different loops in three different immunoassays

The expression level of wild-type hTSH equals 100 for each respective assay.

	ILMA monoclonal		IRMA monoclonal		RIA polyclonal	
	Nichols Institute Diagnostics		DiaSorin Corporation		NHPP, NIDDK, NIH	
WT	100		100		100	
αL1(4K) + βWT	37 ± 9		38 ± 16		43 ± 12	
αL3(3K) + βWT	24 ± 4		23 ± 10		19 ± 8	
αL1(4K)/αL3(3K) + βWT	2 ± 1		2 ± 1		8 ± 1	
αL1(4K) + βL3(3R)	5 ± 1		10 ± 3		6 ± 2	
αL3(3K) + βL3(3R)	3 ± 1		6 ± 1		4 ± 1	
αL1(4K)/αL3(3K) + βL3(3R)	0.2 ± 0.1		0.3 ± 0.1		0.3 ± 0.1	

Four out of seven lysine substitutions generated hTSH superactive analogs, as they showed a 2–6-fold decrease in the hTSH concentration required for half-maximal stimulation when testing cAMP production in the JP09 (Fig. 2A) and JP26 (data not shown) cell lines. Levels of maximal cAMP stimulation were comparable between hTSH wild-type and hTSH analogs. The increase in *in vitro* bioactivity was paralleled by a decrease in the hTSH concentration required for a 50 or 75% displacement of bound radiolabeled bTSH in a porcine thyroid membrane preparation (Fig. 2B). The four mutants showed increases in biopotency in the following order: G73K > N66K > S64K = A81K. Lysine substitutions at positions α65, α67, and α74 did not result in an increase in bioactivity (data not shown), thereby establishing site-specific effects for the gain-of-function mutations.

Analogues with Multiple Mutations—Fig. 3, A and B, demonstrates the increase in *in vitro* bioactivity when lysine substitutions were combined to generate analogs with multiple mutations. Fig. 3A shows the gradual decrease in the hTSH concentration required for half-maximal stimulation in the JP09 cell line for the single mutant G73K, the double mutant (S64K/G73K), and the triple mutant (S64K/N66K/G73K), respectively. Fig. 3B exemplifies the same in the JP26 cell line for A81K, the double mutant (G73K/A81K), and the triple mutant (N66K/G73K/A81K). In contrast to the single lysine substitutions, double and triple lysine substitutions in the αL3 loop did increase maximal cAMP stimulation levels that ranged between 120 and 130% of those found for the respective wild-type preparation.

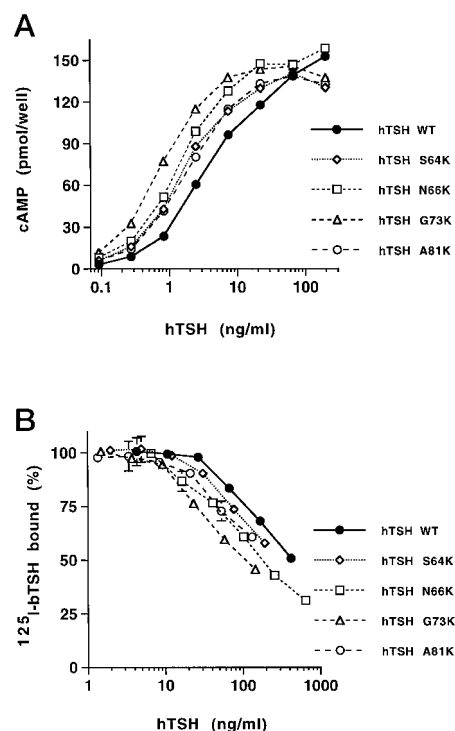


FIG. 2. A and B, single substitutions in the αL3 loop. *In vitro* bioactivity was assessed by cAMP production in the JP09 cell line (A) and receptor binding affinity (B) of αL3 loop analogs with single lysine substitutions at positions α64, α66, α73, and α81. Each data point represents the mean ± S.E. of triplicate determinations in a representative experiment repeated two times. WT, wild type.

Interestingly, we could observe maximum increases in bioactivity with two triple lysine substitutions as the addition of A81K to (S64K/N66K/G73K) and S64K to (N66K/G73K/A81K), respectively, did not result in any further gain-of-function as shown in Fig. 4A. In addition, the triple arginine (N66R/G73R/A81R) displayed equal potency compared with the mutant with three lysine residues, whereas the triple histidine mutant (N66H/G73H/A81H) was significantly less bioactive, thereby providing evidence for residue and charge specificity after introduction of nonconservative amino acid residues (Fig. 4B).

In addition to the assessment of substitutions to basic residues within the αL3 loop as outlined above, we also characterized the effects of selected single substitutions to acidic, negatively charged amino acid residues (glutamic acid, aspartic acid) at positions α64, α66, α73, and α81 as well as one com-

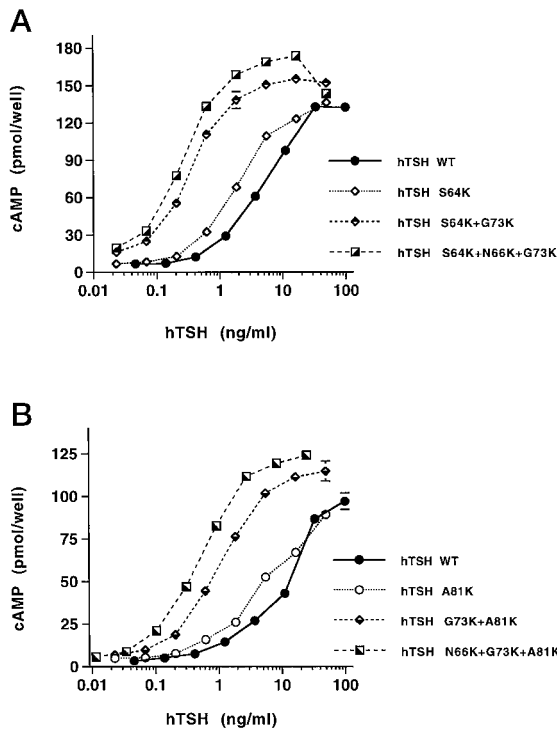


FIG. 3. A and B, combined substitutions in the α L3 loop. *In vitro* bioactivity was assessed by cAMP production in the JP09 (A) and JP26 (B) cell lines. The gradual increase in *in vitro* bioactivity of α L3 loop mutants corresponds to an increase in the number of lysine residues from single to double and triple substitutions. Each data point represents the mean \pm S.E. of triplicate determinations in a representative experiment repeated two times. WT, wild type.

combined mutant with three glutamic acid residues (Fig. 4C). We observed either no change or a decrease in bioactivity as compared with the cAMP stimulation elicited by the wild-type hormone for the single substitutions S64E, N66E, G73D, and A81E as well as for the triple mutant (S64E/N66E/G73E), thereby supporting our concept of a basic charge-dependent enhancement of TSH biopotency. However, a G73E substitution, in contrast to our above findings at positions α 64, α 66, and α 81 for glutamic acid and at position α 73 for aspartic acid, resulted in a 2-fold lower increase in biopotency than the G73K mutation.

When testing the four single superactive lysine substitutions in the α L3 loop in a CHO cell line stably expressing a hTSH receptor construct (26) in which amino acid residues 317–366, unique for the hTSH receptor and not found in the hLH/CG receptor, had been deleted, we observed no loss of superactive potency (Fig. 4D), thereby indicating that the 317–366 region of the thyrotropin receptor does not seem to be involved in mediating superactive effects of the four single lysine substitutions within the α L3 loop. Assessment of cAMP stimulation by the two most potent α L3 loop analogs with triple lysine substitutions also failed to reveal a “loss-of-superagonism” (Fig. 4D).

Cooperative Effects between Peripheral Loops—We chose one of the two previously characterized most potent triple lysine substitutions ((N66K/G73K/A81K), *i.e.* “ α L3(3K)”) for further comparison and combination with two other superactive peripheral β -hairpin loop analogs that had been bioengineered in our laboratory (Fig. 1). We had previously generated an α L1 loop analog with four lysine substitutions at positions α 13, α 14, α 16, and α 20 (*i.e.* “ α L1(4K)”) (21) and an hTSH- β L3 loop analog with three lysine substitutions at positions hTSH- β 58, hTSH- β 63, and hTSH- β 69 (*i.e.* “ β L3(3R)”) (22).

Fig. 5A shows a comparison of the *in vitro* bioactivity for the

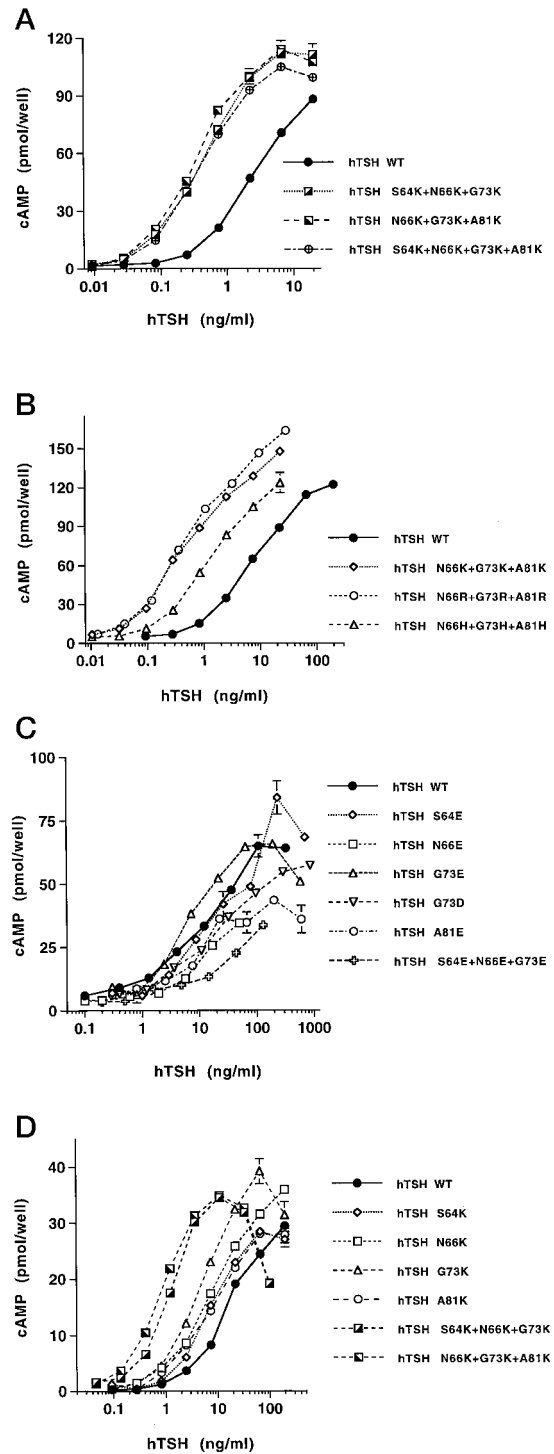


FIG. 4. A–D, combined substitutions in the α L3 loop. *In vitro* bioactivity of triple and quadruple lysine substitutions within the α L3 loop (A) and comparison of triple lysine, arginine, or histidine (B) substitutions are assessed by cAMP production in the JP09 cell line. Each data point represents the mean \pm S.E. of triplicate determinations. *In vitro* bioactivity of selected single and triple acidic (glutamic acid, aspartic acid) amino acid substitutions within the α L3 loop are assessed by cAMP production in the JP09 cell line (C). *In vitro* bioactivity of single and triple lysine substitutions within the α L3 loop are assessed by cAMP stimulation in the hTSHR-D1 cell line (D). WT, wild type.

α L1(4K) and the α L3(3K) analog after co-expression with either hTSH- β wild-type or the hTSH- β L3(3R) analog. We observed a higher degree of cooperative effects for the α L3 loop analog *versus* the α L1 loop analog with the hTSH- β L3 loop analog, because α L3(3K) alone increased cAMP production in

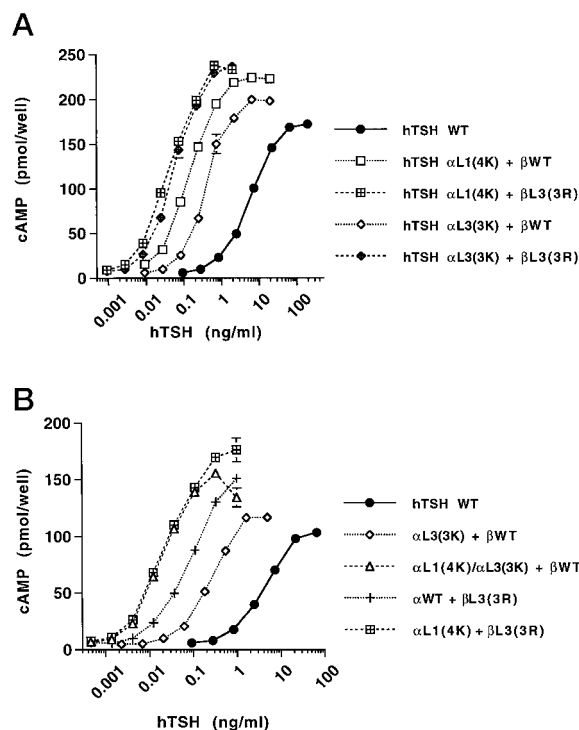


FIG. 5. A and B, combination of two superactive loops. A, comparison of *in vitro* bioactivity after combination of superactive α L1 and α L3 loop analogs with either hTSH- β wild-type (WT) or the hTSH- β L3 loop analog showing different degrees of cooperativity between loops. In analogy, combination of the α L3 loop analog or the hTSH- β L3 loop analog with the α L1 loop analog results in combined mutants with identical increases in *in vitro* bioactivity, although the α L3 loop analog alone is significantly less potent than the hTSH- β L3 loop analog (B). Increases in *in vitro* bioactivity were assessed by cAMP production in the JP09 cell line. Each data point represents the mean \pm S.E. of triplicate determinations in a representative experiment repeated two times.

the JP09 cell line by about 10–20-fold and was therefore significantly less potent than α L1(4K) which showed an increase in *in vitro* bioactivity of about 30–40-fold. However, after combination with the hTSH- β L3 loop analog, the increase in biopotency observed for the α L1-hTSH- β L3 loop combination as well as for the α L3-hTSH- β L3 loop combination was comparable and ranged between 100- and 150-fold. This was also reflected by the receptor binding studies (data not shown).

In analogy to these observations, the combination of the α L3(3K) analog or the hTSH- β L3(3R) analog with the α L1(4K) analog resulted in surprisingly similar increases in *in vitro* bioactivity for the two resulting combinations of superactive loops on either the same (*i.e.* “unipolar” combination) or on opposite (*i.e.* “bipolar” combination) sides of the hTSH molecule (Fig. 1), although the α L3 loop analog alone was significantly less potent than the hTSH- β L3 loop analog (Fig. 5B), so that a higher degree of cooperative effects could again be established for the α L3 loop analog *versus* the hTSH- β L3 loop analog with the α L1 loop analog.

Finally, we combined all three as yet characterized peripheral β -hairpin loops (Fig. 6, A–D). We observed an increase in *in vitro* bioactivity from “single loop” (*i.e.* α L3(3K)) to “double loop” (*i.e.* α L3(3K) + β L3(3R)) and “triple loop” (*i.e.* α L1(4K)/ α L3(3K) + β L3(3R)) analogs (Fig. 6A) and a parallel increase in binding affinity (Fig. 6B). Increases in maximal cAMP levels for combinations of superactive peripheral loops ranged between 150 and 180% of the wild-type preparation. The addition of the α L3(3K) analog to our previously most potent α L1-hTSH- β L3 loop combination was even further enhancing *in vitro*

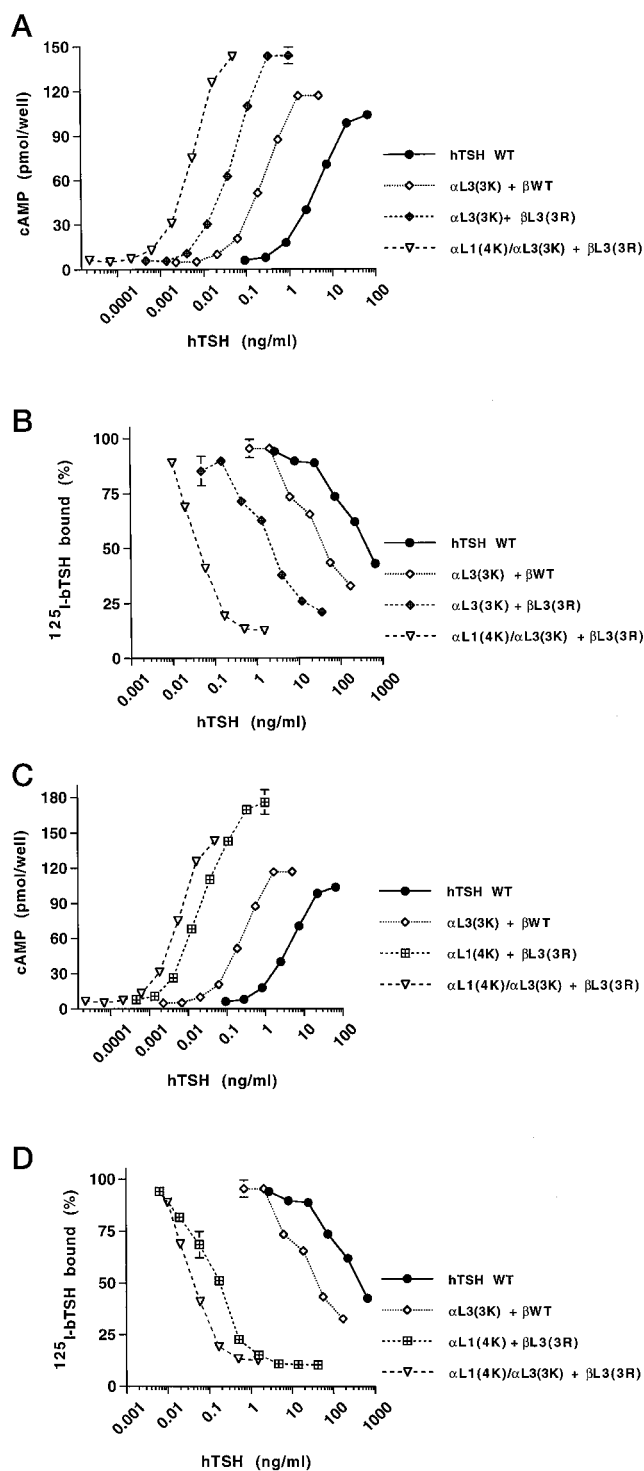


FIG. 6. A–D, combination of three superactive loops. The gradual increase in *in vitro* bioactivity and receptor binding for mutants with one, two, and three superactive peripheral loops are shown (A and B). Combination of the α L3 loop analog with our previously most potent (22) α L1- β L3 loop combination (C and D) results in a further gain in biopotency. Increases in *in vitro* bioactivity were assessed by cAMP production in the JP09 cell line. Each data point represents the mean \pm S.E. of triplicate determinations. WT, wild type.

bioactivity (Fig. 6C) and receptor binding affinity (Fig. 6D), thereby generating the most potent hTSH superactive analog described to date.

Fig. 7 compares the *in vivo* bioactivity for hTSH wild-type, the α L3(3K), and the α L1(4K)/ α L3(3K) superactive analog. Both preparations were highly potent *in vivo* and displayed fold

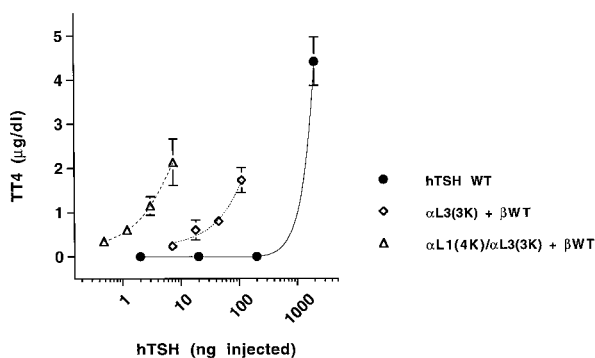


FIG. 7. **In vivo bioactivity.** The comparison of *in vivo* bioactivity for hTSH wild-type (WT) and the α L3(3K) and the α L1(4K)/ α L3(3K) superactive analogs is shown. Increases in *in vivo* bioactivity were assessed by TT4 serum levels 6 h after an intraperitoneal injection of wild-type (WT) hTSH or mutant hTSH analogs. Each data point represents the mean \pm S.E. of groups of 4–5 animals. The experiment was repeated once, and representative results are shown.

increases in *in vivo* bioactivity comparable to those found *in vitro*. Although more extensive *in vivo* studies were beyond the scope of this paper, the levels of hTSH analogs 6 h after intraperitoneal injection suggested no major change in the clearance rate for these analogs. This supported a general applicability of such analogs for *in vivo* stimulation of radioiodine uptake, which will include future tests in rats and primates.

DISCUSSION

In the present study, we have designed mutations in the common α L3 loop of human glycoprotein hormones resulting in gain-of-function thyrotropin analogs. Whereas studies using peptide approaches have suggested a role of this loop in receptor binding (31–33), previous mutagenesis studies have shown no effects or limited “loss-of-function” (34–36). We have modified a recently developed rational design strategy for bioengineering glycoprotein hormone superactive analogs for mutational analysis of the common α L3 loop. Studies from our laboratory and others had suggested a major role of the peripheral β -hairpin loops of cystine knot growth factors in receptor binding (8, 9, 21). We have previously designed hTSH and hCG superactive analogs with significant increases in both receptor binding and signal transduction based on the introduction of positively charged lysine and arginine residues into the common α L1 and the hTSH- β L3 loop (21, 22). The amino acid residues chosen as target sites for site-directed mutagenesis in these studies were delineated from sequence comparisons either between species reflecting different stages of hormone evolution or between different hormones in a given species. Whereas this evolutionary approach proved to be extremely efficient and specific to guide selection of target sites for mutagenesis, it was based on the presence of conserved clusters of positively charged lysine or arginine residues, which could not be seen in amino acid sequence comparisons within the common α L3 loop of human glycoprotein hormones. However, highly variable residues undergoing multiple evolutionary changes could be identified. We hypothesized that three regions with particularly high variability between species (α 64–67, α 73–74, and α 81) may tolerate the introduction of nonconservative amino acid changes, and these were therefore subjected to a lysine-scanning approach. In contrast to alanine-scanning mutagenesis that had been previously utilized in functional studies (37–39), the current strategy might be particularly useful for bioengineering superagonists of glycoprotein hormones because it allowed us to identify three modification permissive domains, and about 60% (*i.e.* four out of seven) of the single lysine substitutions tested in this study

proved to generate gain-of-function mutants.

In preliminary studies we have additionally co-expressed the analogs with single lysine substitutions in the α L3 loop with the hCG β -subunit (data not shown). We observed a differential pattern in the modulation of hTSH and hCG bioactivity by identical lysine substitutions in the common α L3 loop of human glycoprotein hormones, in particular for the A81K substitution, which increased hTSH bioactivity but decreased hCG bioactivity. In addition, biopotency was greater for the G73K than for the N66K substitution when expressed in the context of hTSH, whereas N66K was more potent than G73K when expressed as hCG. Thus, certain mutations in the α -subunit are increasing hTSH bioactivity in a hormone-specific fashion, which can be related to subunit interaction and/or specific hormone-receptor interaction. However, the peripheral location of such mutations suggests that an effect mediated via subunit interaction is less likely. Therefore, it is possible to envision that a specific basic residue in the ligand and an acidic residue in the TSH receptor, not present in the homologous position of the LH receptor, participate in the formation of a new local electrostatic interaction.

We have therefore tested various single and combined lysine substitutions in the α L3 loop for their ability to exceed the cAMP stimulation elicited by the wild-type hormone in the context of a hTSH receptor construct (26) where amino acid residues 317–366, unique for the hTSH receptor and not present in the corresponding structure of the hLH/CG receptor, had been deleted. Interestingly, superactive effects of the various hTSH analogs observed with the wild-type receptor could be reproduced with this receptor mutant, thereby indicating that the deleted hTSH receptor region is not involved in mediating the superactive effects of the α L3 loop superactive analogs. This strategy of assessing the presence or absence of superactive effects originally characterized for the wild-type receptor with different receptor chimeras or receptor mutants constitutes a separate tool in the analysis of ligand-receptor interaction of glycoprotein hormones. By first screening for possible regions of interaction with overlapping libraries of receptor chimeras, it should be possible to exclude significant portions of the receptor as contact sites for a given mutated ligand. In a second step, certain regions of interest can then be subjected to a more detailed analysis by characterizing defined receptor mutants where a limited number of amino acid residues have been changed. Finally, a charge reversal technique switching individual oppositely charged residues between the ligand and the receptor can be used assuming that this should reestablish the original superactive interaction. Therefore, it should be possible to pinpoint specific interaction sites between hTSH and the hTSH receptor with the help of superactive thyrotropin analogs.

As in our previous studies, gain-of-function effects were highly site-specific, as only introduction of lysine residues at positions α 64, α 66, α 73, and α 81, but not at positions α 65, α 67 and α 74, resulted in the generation of superactive analogs. They were also residue- and charge-specific, as a mutant with three lysine or arginine (*i.e.* strongly basic) residues at positions 66, 73, and 81 showed similar increase in biopotency and was significantly more potent than a triple histidine (*i.e.* weakly basic) analog. A maximal increase in biopotency was reached by combination of three basic residues within the α L3 loop, in contrast to our previous observation where the most potent α L1 loop analog had been engineered by introduction of four basic substitutions (21).

Two published crystal structures of deglycosylated hCG, the homology model of hTSH, and epitope mapping studies indicated that amino acid residues α 64, α 66, α 73, and α 81 in the

α 64–81 region are surface-oriented and not involved in hydrogen bonding with the TSH β -subunit (5, 6, 21, 33, 40). An introduction of basic residues in such locations enhanced the hormone-receptor interaction. However, α 68, α 70, α 71, α 74, and α 76 contribute to the formation of a hydrophobic patch between two α -subunit loops. Accordingly, an F74K mutation did not show any effect on TSH binding to its receptor.

The observation that the introduction of negatively charged glutamic acid or aspartic acid residues at these positions resulted in all but one mutant characterized in either no change or a decrease in biopotency is further supporting our hypothesis of a modulation of *in vitro* hTSH analog bioactivity by alteration of electrostatic interactions between ligand and receptor. The slight increase of hTSH bioactivity demonstrated in a single mutant with an amino acid substitution to glutamic acid at position α 73, however, may be attributed to other effects than a change in electrostatic interactions with the receptor, *i.e.* that the effects of introducing negative charges in the ligand may not always depend on electrostatic repulsion. Considering the fact that an amino acid substitution to aspartic acid at the same position did result in a decrease in biopotency, the different lengths of the amino acid side chain may also play a role in ligand-receptor interaction. In addition, position α 73 is in close proximity to the tip of the α L3 loop, with the most exposed location of all amino acid residues investigated in this study which might account for considerable conformational flexibility after introduction of nonconservative amino acid changes. Finally, from this study, hydrophobic interactions or a modification of hydrogen bonding cannot be excluded as additional events modulating the overall change in bioactivity observed in the course of our experiments. More detailed studies in the future will be needed to specifically address these issues.

The assessment of *in vitro* bioactivity of the various α L3 loop mutants by measurement of cAMP production as well as binding studies were performed under isotonic low salt conditions. Although we did not study the effect of low salt *versus* normal salt conditions on the *in vitro* bioactivity of analogs with the α L3 loop mutations, we have previously compared the effect of low salt *versus* physiological salt concentration in the assessment of *in vitro* bioactivity of superactive TSH analogs with mutations in the α L1 and β L3 loop (22). These studies revealed only small differences in fold increases in *in vitro* bioactivity under different (0–0.9% NaCl) ionic conditions, which could be demonstrated to be comparatively minor when considering the fold increases elicited between wild-type and superactive mutant hormone. Furthermore, we have previously tested the effect of α L1 and β L3 analogs on proliferation of FRTL-5 cells and thyroid hormone production in cultured human thyroid follicles using physiological salt conditions (21). Again, only small differences in fold increases were noted in comparison to the standard isotonic low salt conditions of the cAMP bioassay.

Parallel to our previous studies, we observed cooperative effects when single basic substitutions within one peripheral β -hairpin loop were combined. These findings confirmed that additional electrostatic interactions between the ligand and the receptor resulting from the introduction of additional lysine residues into the ligand are not only site-specific but also directly cooperative indicating that they are not likely resulting from distant conformational changes.

The combination of the three as yet characterized superactive peripheral β -hairpin loops of human thyrotropin allowed us to extend our studies to the analysis of cooperative effects between loops. The higher degree of cooperativity between the α L3 *versus* the α L1 loop analog with the hTSH- β L3 loop analog and the surprisingly similar increases in biopotency between unipolar and bipolar combinations suggest that two modified

superactive peripheral loops in closer proximity may exhibit higher cooperativity than two more spatially distant loops, further indicating considerable conformational flexibility of both the ligand and the hTSH receptor at the ligand-receptor interface. In addition, the fact that the combination of the superactive α L1, α L3, and the hTSH- β L3 loop analogs was more potent than any combination of two loops supports the concept that all three engineered loops are involved in receptor binding and signal transduction.

The lysine-scanning approach first used for this study as an extension of our previous evolutionary approach in the rational design of superactive analogs of human thyrotropin constitutes a new, separate tool in the design of glycoprotein hormone superagonists. Moreover, the results of this highly efficient approach support the concept that it is possible to increase the electrostatic component of hormone-receptor interaction by introduction of positively charged amino acid substitutions into the ligand either resulting in a recruitment of *de novo* binding sites not used by the natural hormone or leading to higher binding affinity in preexisting interaction domains.

Superactive analogs of hTSH are not only providing new insights into hTSH structure-function relationships but also have a wide range of potential diagnostic and therapeutic clinical applications. Wild-type recombinant human thyrotropin has been under extensive basic and clinical study (41, 42) as diagnostic preparation for the follow-up of patients after thyroidectomy for thyroid carcinoma. Our design strategy for superactive analogs of human thyrotropin combining evolutionary and lysine-scanning approaches provides the bioengineering tools for developing a second generation recombinant thyrotropin with major improvements in clinical efficacy and therefore enhanced diagnostic and therapeutic utility. The principles derived from these studies are applicable not only to other glycoprotein hormones, but may also be useful for bioengineering other members of the cystine knot growth factor superfamily.

Acknowledgments—We are indebted to Dr. Basil Rapoport for supplying us with the hTSHR-D1 construct. We thank Dr. Christine Leitolf for valuable help and indispensable support during all stages of this study, especially during the preparation and review of the manuscript. We also thank Dr. Rasa Kazlauskaitė for technical assistance and general support.

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